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Accuracy of immunofluorescence in the diagnosis of Primary Ciliary Dyskinesia

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Statement of contribution:

AS, CH and AB designed the study. EF, KK, SO and AS consented patients, conducted light microscopy, collected nasal brushings and prepared slides. EF and AS conducted IF staining and analysis. MD conducted light and electron microscopy. HM, MP and JS provided genotyping. AS and EF analysed the data. AS, CH and AB drafted the manuscript. All authors contributed to manuscript drafts and preparation. AS is custodian of the data and takes responsibility for its accuracy.

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At a Glance Commentary: Scientific Knowledge on the Subject

Primary Ciliary Dyskinesia is a genetically heterogeneous chronic condition. Early diagnosis is key to attenuating disease progression by implementation of appropriate medical management. Currently diagnosis requires expensive and complex equipment

What This Study Adds to the Field

This study validates the clinical use of a panel of commercially available antibodies to diagnose Primary Ciliary Dyskinesia by immunofluorescence, a simpler, more widely available, cost effective alternative to current confirmatory diagnostic tests.

Immunofluorescence is a useful diagnostic test for PCD, reduces the need for repeat biopsies, and improves turnaround time without compromising diagnostic accuracy.

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"This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org"

ABSTRACT

Rationale The standard approach to diagnosis of primary ciliary dyskinesia (PCD) in the UK consists of assessing ciliary function by high-speed-microscopy and ultrastructure by election microscopy, but equipment and expertise is not widely available internationally. The identification of bi-allelic disease causing mutations is also diagnostic, but many disease causing genes are unknown, and testing is not widely available outside the USA. Fluorescent antibodies to ciliary proteins are used to validate research genetic studies, but diagnostic utility in this disease has not been systematically evaluated.

Objectives Determine utility of a panel of six fluorescent labelled antibodies as a diagnostic tool for PCD.

Methods Immunofluorescent labelling of nasal brushings from a discovery cohort of 35 patients diagnosed with PCD by ciliary ultrastructure, and a diagnostic accuracy cohort of 386 patients referred with symptoms suggestive of disease. The results were compared to diagnostic outcome.

Measurements and Main Results Immunofluorescence correctly identified mislocalised or absent staining in 100% of the discovery cohort. In the diagnostic cohort immunofluorescence successfully identified 22 of 25 patients with PCD and normal staining in all 252 in whom PCD was considered highly unlikely. Immunofluorescence additionally provided a result in 55% (39) of cases which were previously inconclusive. Immunofluorescence results were available within 14 days, costing $187 per sample compared to electron microscopy (27 days, cost $1452).

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Conclusions Immunofluorescence is a highly specific diagnostic test for PCD, and improves the speed and availability of diagnostic testing, however, sensitivity is limited and immunofluorescence is not suitable as a stand-alone test.

Abstract word count: 250

Key words: Cilia, Electron microscopy, Antibody

INTRODUCTION

Primary Ciliary Dyskinesia (PCD) affects approximately 1 in 15,000 of the population. Its manifestations are caused by defective ciliary beating and reduced mucociliary clearance. Diagnosis is frequently delayed, and delay is associated with significant impairment of lung function. Diagnostic delay is related to two factors: the non-specificity of symptoms (cough, rhinitis) and the lack of an easy and widely available diagnostic test for the condition [1]

The diagnostic pathway for PCD typically includes measurement of nasal nitric oxide and nasal brush biopsy for light and electron microscopy. Light microscopic assessment of ciliary function on cells ex-vivo is by high speed video analysis of the frequency and pattern (waveform) of cilia movement. Electron microscopy allows visualisation of the ultrastructure of cilia and can often provide a definitive diagnosis [1]. All these tests require sophisticated equipment and considerable expertise, and in consequence are only available in very few centres. Genetic testing for PCD is also increasingly used, but there are at least 200 potential motile cilia genes which are widely scattered through the human genome. To date, more than 30 disease-associated mutations have been identified, which are estimated to account for 60-65% of known cases [1,2,3].

Immunofluorescence allows indirect imaging of target proteins by fluorescent or confocal microscopy using specific antibodies with fluorescent tags. The use of different tags for double labelling allows the co-localisation of proteins to be determined. Immunofluorescence for the diagnosis of PCD was first described in 2005 and was subsequently recommended in the ERS expert consensus statement for diagnosis and treatment of PCD [4,5]. Despite this recommendation limited availability of validated antibodies and lack of evidence for the

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diagnostic accuracy of the technique has limited its use. Immunofluorescence has been used extensively in PCD research in confirming protein absence due to genetic mutations. A number of antibodies to proteins defective in PCD have been developed and validated. These include DNAH5 (an outer dynein arm heavy chain) [4,6], DNALI1 (an inner dynein arm light chain)[6,7], GAS8 (a component of the nexin -dynein regulatory complex) [7] and RSPH4A, RSPH9 and RSPH1 (components of the radial spoke)[8]. These antibodies represent markers of the four key ultrastructural abnormalities in PCD. Detected by electron microscopy these defects are the end products of multiple gene defects (Table 1 and Figure 1). By immunofluorescence the absence or mislocation of a single protein can allow the effects of mutations in multiple genes to be detected (Table 1). We hypothesised that immunofluorescence using a panel of antibodies would be a useful diagnostic test for PCD, and aimed to assess this in the clinic in a large cohort of patients with possible PCD referred for diagnostic testing.

METHODS

Subjects

Discovery cohort- Nasal brushings were analysed from a cohort of 35 patients with a known PCD ultrastructural defect (thus excluding 30% of PCD patients with normal ultrastructure)[9].

Diagnostic accuracy cohort- Nasal brushings were analysed from 386 patients sequentially referred to the Royal Brompton Hospital for PCD diagnosis. Referrals were due to symptoms suggestive of PCD such as situs inversus, neonatal respiratory distress, bronchiectasis, recurrent chest infections, rhinosinusitis and otitis media (listed in OLS supplementary table 1).

Diagnosis of PCD

All patients underwent a standardised diagnostic protocol regularly audited across 3 UK PCD centres which form a national specialised service (Leicester Royal Infirmary, Southampton Hospital and Royal Brompton Hospital) [1]. This consisted of six assessments as follows:

1. Assessment for symptoms suggestive of PCD (n= 378/386; 8 external samples had a limited clinical history).
2. Nasal Nitric Oxide measurement – In children >4 years. Two readings from each nostril were taken during a breath hold manoeuvre using a chemiluminescent analyser LR2000 (Logan Research, Rochester, UK) [10] (n= 129/386)
3. High speed video microscopy for cilia beat frequency and ciliary beat pattern measured at 37°C using a 100x objective [11] (n=386/386)
4. Quantitative transmission electron microscopy of ciliary ultrastructure [12] (n=208/386)
5. Air liquid interface culture of difficult samples and repeat light and electron microscopy [13] (n=115/386)
6. Genotyping from blood sample in patients with likely PCD based on positive results from at least two of the other investigations (n=16/386)

In the absence of an established gold standard, a diagnosis of PCD was made following a review of clinical and laboratory findings in a monthly multidisciplinary meeting led by a consultant clinician with expertise in PCD. Further details of diagnostic decision making pathways are described in the online supplement.

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Experimental methods

Methodological details are provided in the online data supplement [4]. All slides were double labelled with acetylated alpha tubulin (T7451, Sigma Aldrich) in order to visualise cilia. Antibodies of interest were used in a two step protocol. All nasal brushings were assessed for Panel 1: DNAH5 (HPA037470), DNALI1 (HPA053129), RSPH4A (HPA031196). A second round of antibodies were used in selected cases: Panel 2: RSPH9 (HPA031703), RSPH1 (HPA017382) or GAS8 (HPA041311)

Slides were scanned under a fluorescent microscope for ciliated cells at x40 magnification to identify acetylated tubulin. In each ciliated cell, the co-localised ciliary protein of interest was assessed in a second channel. If there was visual co-localisation of the antibody label with acetylated tubulin, the target protein was considered present. If more than seven of ten ciliated cells were clearly labelled with the target protein antibody the sample was considered normal for that protein. PCD was defined as diagnosed when on duplicate slides all ciliated cells observed had absent staining of the target protein from the axoneme or staining consistently isolated to the distal or proximal portion of the cilia. Insufficient (<10 cells observed) and inconclusive slides were repeated. If the first slide was insufficient or inconclusive but the second assessment was normal the result was considered normal (n=71).

Statistical methods

The number of patient results reviewed was based on a 10% prevalence of PCD in the population tested. Power calculations predicted 271 patients with a confirmed diagnosis of 'PCD' or 'PCD highly unlikely' would be required for 95% confidence. Results were analysed in graph pad prism 5 and a p value <0.05 was considered significant.

ETHICS

This study was conducted according to the recommendations of the Declaration of Helsinki. The protocol was approved by ethical review committee and written consent was obtained from subjects or their parent/guardian.

RESULTS

Immunofluorescence can be used to confirm a diagnosis of PCD

Antibodies were tested on nasal brushings from a discovery cohort of 35 patients with PCD with a confirmed ultrastructural ciliary defect. Results shown in Table 2 demonstrated a complete agreement between the absence of structure by electron microscopy and absence of associated protein by immunofluorescence.

Diagnostic accuracy of immunofluorescence in the diagnostic accuracy cohort

The immunofluorescence technique successfully demonstrated an absence of target proteins from the axoneme in 22 of 25 patients with a diagnosis of PCD as defined above. Normal results were obtained in all 252 patients who were considered highly unlikely to have PCD.

Figure 2 shows an example of immunofluorescent staining of ciliated epithelial cells from two patients who were ‘PCD-positive’ and a patient who was ‘PCD-highly unlikely’. Results of the diagnostic tests and immunofluorescence tests are shown in Tables 3 and 4. Immunofluorescence identified PCD protein defects in all patients who had an identifiable ultrastructural defect by electron microscopy. The number of patients in which PCD was confirmed by immunofluorescence was the same as that of electron microscopy.

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Three patients who were diagnosed as 'PCD- positive' were not identified using the immunofluorescence protocol and were examined in closer detail. Genetic tests in two patients showed mutations in *DNAH11*. Both patients had a beat pattern consistent with this defect (hyperfrequent, stiff and static) on more than one biopsy and nasal NO <77nl/min. One patient had consanguineous parents and was homozygous for a nonsense mutation (c.3380G>A , p.Trp1127*) the other had heterozygous nonsense changes (c.5506C>T , p.Arg1836* and c.5636T>A p.Leu1879*). The third individual was also from consanguineous parentage and was homozygous for a frame shift mutation in the *HYDIN* gene (c.2196dupT, p.Y372fs). The cilia ultrastructure in these three patients was considered normal by standard electron microscopy. Electron tomography from the patient with the *HYDIN* mutations showed absence of the c2b central pair projection [14].

Insufficient and inconclusive samples

Immunofluorescence as part of the diagnostic pathway could reduce the requirement for repeat nasal brushing.

In 71 patients a conclusive multidisciplinary diagnosis was not made on first sampling due to insufficient tissue for light and/or electron microscopy. The immunofluorescence protocol required fewer ciliated cells and was able to produce a definitive result for 55% (39/71) of these cases. Two of these cases showed an absence of DNAH5 by immunofluorescence and this was supported subsequently by electron microscopy on a repeat biopsy as an outer dynein arm defect. The remaining 37 samples, in which immunofluorescence testing was normal, were considered 'PCD highly unlikely' on second testing either by repeat nasal brushing (n=31) or by culturing the original sample at air liquid interface (n=6). Once a sufficiently ciliated sample had been acquired by repeat brushing or cell culture the high

speed video and electron microscopy as per the original protocol. None of these patients underwent genetic testing.

Insufficient and inconclusive results were sometimes obtained by immunofluorescence when a multidisciplinary result was conclusive

In 42 samples in which a multidisciplinary diagnosis was made there were not enough cells for immunofluorescence. In a further 32 samples in which a multidisciplinary diagnosis was made the immunofluorescence result for one or more antibody was inconclusive on more than one occasion. Sixty nine of these patients (represented by 71 samples) did not have PCD, however, two were diagnosed with PCD at the multidisciplinary meeting. One of these two patients yielded a sample which was insufficient for immunofluorescence, light microscopy and electron microscopy but was found to have a mutation in the *CCNO* gene (c.258_262dupGGCCC, p.Gln88Argfs*8). This patient was related to a previously genetically characterised family with reduced generation of multiple motile cilia [22]. The second patient with PCD had DNAH5, DNALI1 and GAS8 present, however, inconclusive results were obtained by immunofluorescence for RSPH4A on two separate slides and the other radial spoke head proteins RSPH1 and RSPH9 were absent. This patient had a circular beat pattern on light microscopy and a transposition /central pair complex defect detected by electron microscopy. These insufficient and inconclusive patients were excluded from the diagnostic accuracy analysis. In thirty five patients a diagnosis of ‘PCD positive’ or ‘PCD highly unlikely ’ could not be reached during the study period.

We further investigated factors relating to insufficient and inconclusive immunofluorescent results. Both blood and mucus in the sample appeared to be confounding factors. In 63% of samples with inconclusive results by immunofluorescence viscous mucus was surrounding

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the cilia on the high speed video light microscopy assessment compared to 46% of conclusive samples ($p<0.05$). In 25% of samples insufficient for immunofluorescence blood was seen in the sample compared to 8% in sufficient samples ($p<0.01$). The cause of this relationship is unknown. We hypothesise that increased blood could prevent cells from attaching to the slides thus reducing available sample for immunofluorescence. Alternatively an increase of blood in a sample could represent damaged mucosa denuded of cilia as a result of a recent infective or inflammatory process

Samples were deemed insufficient or inconclusive if a result could not be obtained for one or more antibodies. Thirty percent were inconclusive or insufficient for just one antibody, 16% for two antibodies and 54% for three or more antibodies. Couriered and cultured samples showed similar results compared to nasal brushings taken on site.

Time for results to be available and cost of investigation

The effectiveness of immunofluorescence for diagnosis was compared to electron microscopy. The turnaround time, defined as time from the sample being taken to the results being reported was median 14 days (range 1 - 40) for immunofluorescence compared to 27 days (range 9 - 61) for electron microscopy $p<0.05$. Additionally a cost assessment exercise which included staff hours, equipment running costs and consumables showed that the cost per sample was $187 for immunofluorescence and $1452 for electron microscopy. The assessment did not include the purchase and set up of equipment required or brushes for obtaining cells, as surplus cells from video microscopy are used for both techniques.

DISCUSSION

Using immunofluorescence all cilia structural defects were correctly identified in an initial discovery cohort of patients with a known electron microscopy based ultrastructural diagnosis of PCD. In a cohort subsequently tested for diagnostic accuracy consisting of 386 consecutively referred cases immunofluorescence successfully identified 22 of 25 patients with a multidisciplinary diagnosis of PCD and 252 of 252 in whom the diagnosis was considered highly unlikely. The accuracy of immunofluorescence was the same as that of electron microscopy. Immunofluorescence failed to identify 12% PCD cases in the present study, and may well miss more cases in populations with a different genetic makeup, and is therefore not suitable for use as a stand-alone test. This report does however provide strong evidence for introducing this test into clinical practice as part of the diagnostic armamentarium for PCD.

The main strength of the immunofluorescence technique is the cost reduction and improved turnaround time relative to electron microscopy to confirm a diagnosis of PCD. The cost of the test and the basic equipment and the simplicity of the test may allow improved accessibility to a wider population of patients. The immunofluorescence technique also works on small samples where there are too few cells to process for electron microscopy analysis. In the diagnostic cohort an additional 34 cases could have been diagnosed with the inclusion of immunofluorescence before electron microscopy in the diagnostic pathway.

The strengths of this study include that it has been conducted in the UK clinical setting which follows a nationally audited and standardized algorithm for PCD diagnosis giving a realistic indication of how this test performs. Other strengths include the use of discovery and validation cohorts, with large numbers in this latter group in particular.

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The foundation for pursuing this study, and an acknowledged problem in the field of PCD diagnostics, is the lack of gold standard for the diagnosis of PCD. It is difficult to exclude a diagnosis of PCD due to poor sensitivity of electron microscopy and genetic testing and the poor specificity of nasal nitric oxide [15] Despite use of a multidisciplinary diagnosis to maximize diagnostic capability fifteen patients had an indeterminate diagnosis (a situation also seen in cystic fibrosis, for example). Our approach to ruling in or ruling out a diagnosis of PCD is certainly not perfect. There are multiple variables that could lead to false-negatives, including: 1) The lack of standardized clinical phenotyping, whereby diagnostic decisions are made by a monthly multi-disciplinary meeting (an approach which, however, is used in interstitial lung disease for example [16]), using clinical judgment. There are two recently published approaches to define specific criteria for a clinical phenotype that have significant predictive power for a subject having PCD which could be used in future studies [17,18] , although both also have imperfect sensitivity and specificity 2) lack of ciliary EM defects in ~30% of PCD patients [9], and many of these cannot be diagnosed by immunofluorescence; 3) the limitations of high-speed video-microscopy; 4) only a minority of patients (129 of 386) were tested by nasal nitric oxide, although normal nitric oxide does also not rule out PCD [10]; and, 5) there is limited genetic testing (16 of 386), and in any event, many PCD genes are currently unknown. Thus, it seems logical to conclude that an unknown number with indeterminate diagnosis will have PCD, and exclusion of these cases from this study will have exaggerated the perceived accuracy of the immunofluorescence test. Nonetheless, our results suggest that immunofluorescence is a useful part of diagnostic testing for PCD.

Immunofluorescence was unsuccessful in a number of cases. These cases were excluded from the analysis. This resulted in a case with reduced generation of motile cilia (*CCNO*) being excluded from this study as there were insufficient cilia to perform the immunofluorescence test. Exclusion of unsuccessful immunofluorescence analysis is another factor which could lead to an over estimation of the testperformance. Test failure again highlights that immunofluorescence should not be used as a stand-alone test, as a number of patients would need further repeat investigations. We identified blood and mucus to be associated with failed testing and suggest further methodology improvements could focus on these factors.

The major limitation of the immunofluorescence technique is that the antibodies used are directed to specific proteins of interest and therefore defects in unrelated proteins will be missed. The selected six antibody panel represents the major ultrastructural defects and end products of multiple gene defects, however three cases were still missed. The three cases missed in this study had biallelic mutations in *DNAH11* and *HYDIN*. This is expected since previous publications have demonstrated the outer and inner dynein arms, nexin links and radial spokes are present in patients with these defects [14,19]. Normal ultrastructure cases such as these are also the most likely to be missed using our diagnostic protocol due to limited genetic studies. Despite patients with DNAH11 mutations making up 8% of PCD positive patients in the validation cohort, which is in keeping with predicted numbers of 6-9% from genetic screening programs, it is possible that additional genetic testing may lead to the diagnosis of further PCD cases [19].

New reliable antibodies to DNAH11 have recently been reported and will benefit diagnosis and research in this area [20]. Antibodies to HYDIN proteins are commercially available however we have not been able to validate the use sufficiently for use in PCD diagnosis, nor

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is there any report in the literature of the successful use of these antibodies to identify PCD. Furthermore, the cilium consists of over 200 proteins and patients with partial defects or missense mutations have been shown to have normal immunofluorescence results, it is therefore likely that other cases of PCD and reduced ciliation will be missed by the current immunofluorescence technique and the panel of antibodies will need to be increased in the future as the field of PCD genetics expands [20].

Given the similar diagnostic rate as electron microscopy we envisage that immunofluorescence could be useful where transmission electron microscopy equipment or expertise is not available. In specialist diagnostic centers where microscopy facilities are available the technique could be added to the diagnostic protocol to improve diagnostic success and reduce the number of electron microscopy tests required. Application of the immunofluorescence technique in this study was incorporated into our PCD diagnostic pathway following light microscopy assessment of cilia beat frequency and waveform. Firstly, a core panel of three antibodies was applied and then a second panel based on the primary panel and the light microscopy findings. This two-step protocol allows cost, time and tissue savings but introduces a selection bias. In this study three patients were diagnosed using the second panel by the GAS8 antibody. As absence of DNALI1 always co-existed with absence of GAS8 or DNAH5 we suggest DNALI1 might be substituted for GAS8 in the first panel. Recent data show that use of a RSPH9 antibody may detect a broader range of central pair complex defects than RSPH4A [21]. Therefore, GAS8, DNAH5 and RSPH9 might be a more appropriate selection for the first panel.

In conclusion immunofluorescence is a useful diagnostic test for PCD, reduces the need for repeat biopsies, and improves turnaround time without compromising diagnostic accuracy. We suggest it should be included in the routine PCD diagnostic pathway.

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FIGURE LEGENDS

Figure 1: Diagram of the ultrastructure of a motile cilium in transverse section. Labels indicate ultrastructural features targeted by immunofluorescence with corresponding antibodies **(bold text)**.

Figure 2; Workflow diagram indicating the pathway of patients from referral to diagnosis during the study period. Numbers in bold indicate the number of patients at each stage of the study (not the number of samples). Excluded groups are indicated in red.

Figure 3: Example results in PCD and 'PCD highly unlikely' samples for three antibodies used in the immunofluorescence panel. The left column shows the cell nucleus in blue by DAPI, the next column shows presence of cilia on the cell in green by acetylated tubulin. The third column shows the protein of interest in red and the final column a merged image of the three channels. In the merged image yellow suggests co-localisation and presence of the protein of interest, green suggests absence of the protein of interest. The top images show DNALI1 (an inner dynein arm component), the middle images DNAH5 (an outer dynein arm component), and the bottom RSPH4A (a radial spoke head component)

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TABLES

Ultrastructural Defect	Gene	Immunofluorescence
Outer dynein arm defect	*DNAH5* *DNAI1* *ARMC4* *CCDC114* *TXNDC3 (NME8)* *DNAI2* *DNAL1* *CCDC151*	DNAH5 Absent
Inner and outer dynein arm defect	*C21orf59* *ZYMND10* *CCDC103* *DNAFF2 (KTU)* *DNAFF1 (LRRC50)* *LRRC6* *DNAFF3 (C19orf31)* *HEATR2* *DYX1C1* *SPAG1*	DNAH5 Absent DNALI1 Absent
Central complex / transposition defect	*RSPH4A* *RSPH9* *RSPH1* *RSPH3*	RSPH9, RSPH4A,RSPH1 absent RSPH9 absent RSPH9, RSPH1 absent All present
Microtubular disorganisation with loss of inner dynein arm	*CCDC39* *CCDC40*	DNALI1 Absent GAS 8 Absent
Microtubular disorganisation with present inner dynein arms	*CCDC65* *CCDC164* *GAS8*	GAS 8 Absent
Normal ciliary ultrastructure	*HYDIN* *DNAH11* *OFD1* *RPGR*	All present
Ciliary 'aplasia'	*CCNO* *MCIDAS*	All present DNAH5& LI1Absent

Table 1: The major classes of ultrastructural defects seen in PCD and the gene associated with each defect (former gene name is shown in brackets). Column three shows the assumed coverage of the six antibodies investigated in this study over the known ultrastructural and gene defects. Absent = complete or partial absence from the axoneme

Electron microscopy defect	Absent or mislocalised antibody	Number of patients with PCD tested (total n=35)
Outer dynein arm defect	DNAH5	14
Outer and inner dynein arm defect	DNAH5 DNALI1	10
Inner dynein arm and microtubular disorganisation defect	DNALI1 GAS8	7
Transposition defect / central pair absence	RSPH4A RSPH9 RSPH1	4

Table 2: Immunofluorescence antibody results from 35 patients with primary ciliary dyskinesia due to a known ultrastructural defect

		Standard diagnosis	
		PCD positive	PCD negative
Immunofluorescence diagnosis	PCD positive	22	0
	PCD negative	3	252

Table 3: Diagnostic outcome table comparing immunofluorescence technique to the standard diagnostic approach in 271 patients referred to a national referral centre for investigation into symptoms suggestive of primary ciliary dyskinesia (PCD)

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Multidisciplinary diagnosis	Electron microscopy		Immunofluorescence					
			Panel 1	Panel 2				
	n		DNAH5	DNALI1	RSPH4A	GAS8	RSPH9	RSPH1
Normal n= 208	208	Normal ultrastructure *97% both dynein arms present (+/- 7%)* *89% normal microtubular arrangement (+/- 6%)*	present	present	present	ND	ND	ND
PCD n= 25	8	IDA and ODA defect	**absent**	**absent**	present	present	ND	ND
	8	ODA defect	**absent**	present	present	ND	ND	ND
	2	IDA and MTD defect	present	**absent**	present	**absent**	ND	ND
	1	Transposition defect	present	present	**absent**	present	**absent**	**absent**
	3	MTD defect	present	present	present	**absent**	present	present
	3	Normal ultrastructure	present	present	present	present	present	present

Table 4: Comparison of results from, electron microscopy and immunofluorescence testing of nasal brushings from patients referred due to clinical symptoms suggestive of primary ciliary dyskinesia. Results from all diagnostic tests are shown in supplementary table 1

Figure 1

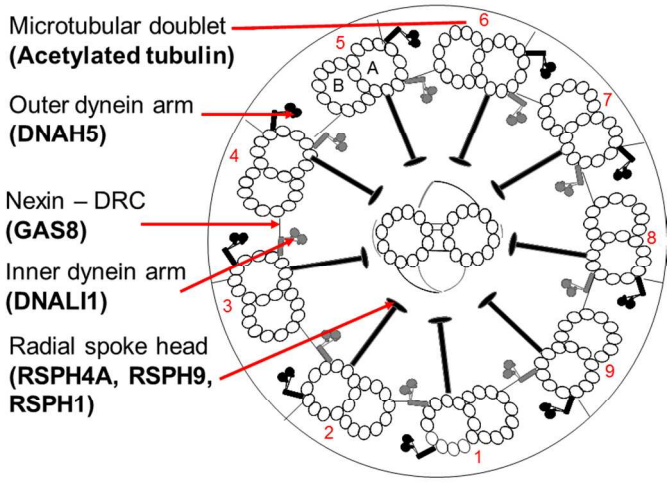

Figure 1: Diagram of the ultrastructure of a motile cilium in transverse section. Labels indicate ultrastructural features targeted by immunofluorescence with corresponding antibodies (bold text).

346x559mm (72 x 72 DPI)

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Figure 2

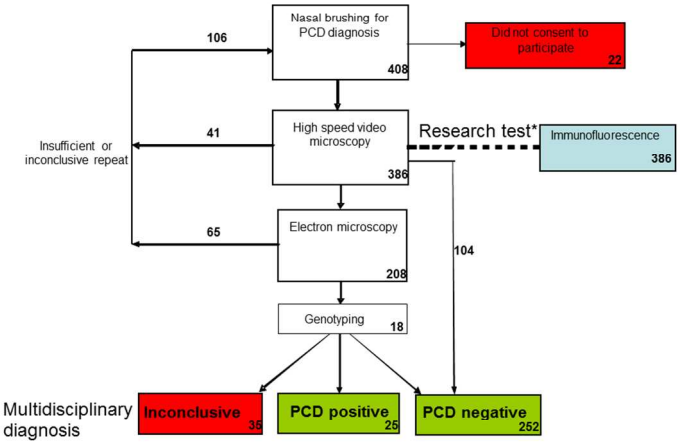

Figure 2; Workflow diagram indicating the pathway of patients from referral to diagnosis during the study period. Numbers in bold indicate the number of patients at each stage of the study (not the number of samples). Excluded groups are indicated in red.

346x559mm (72 x 72 DPI)

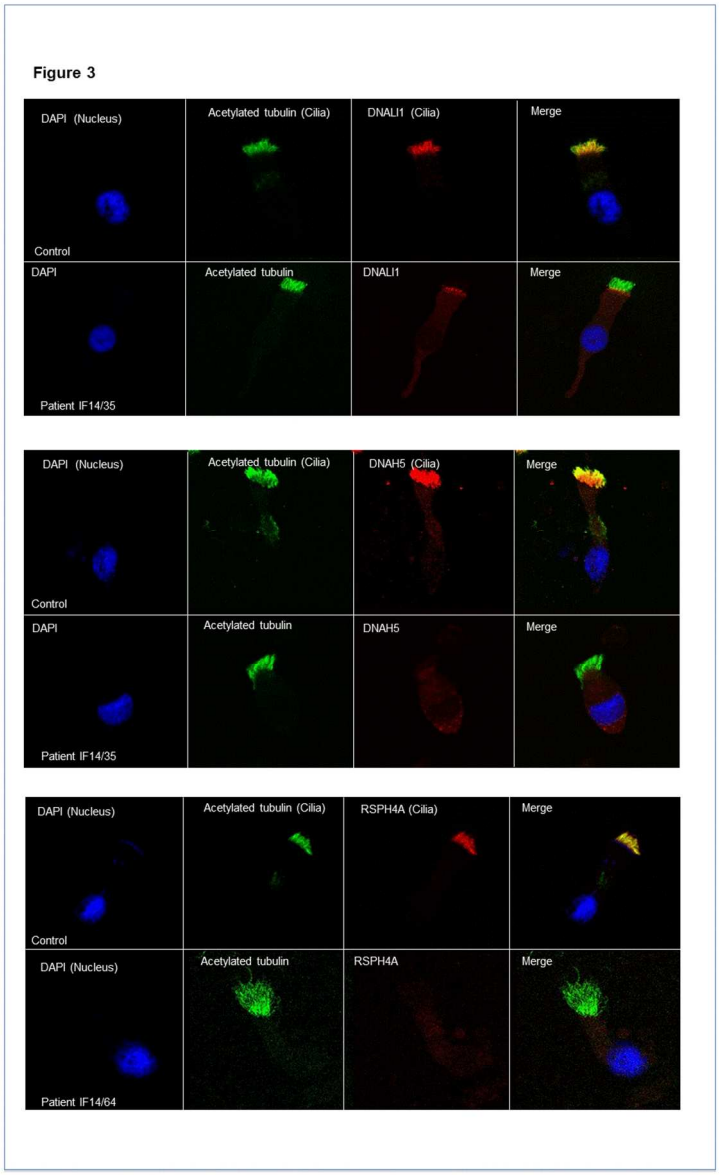

Figure 3: Example results in PCD and 'PCD highly unlikely' samples for three antibodies used in the immunofluorescence panel. The left column shows the cell nucleus in blue by DAPI, the next column shows presence of cilia on the cell in green by acetylated tubulin. The third column shows the protein of interest in red and the final column a merged image of the three channels. In the merged image yellow suggests co-localisation and presence of the protein of interest, green suggests absence of the protein of interest. The top images show DNALI1 (an inner dynein arm component), the middle images DNAH5 (an outer dynein arm component), and the bottom RSPH4A (a radial spoke head component)

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Supplementary methods

Subjects

Sequential nasal brushings were analysed from 386 patients referred to the paediatric department at the Royal Brompton Hospital for PCD diagnosis. Referrals were due to symptoms suggestive of PCD such as situs inversus, neonatal respiratory distress, bronchiectasis, recurrent chest infections, rhinosinusitis and/or otitis media. The prevalence of symptoms for which patients were referred are shown in supplementary table one.

Inclusion criteria: Consecutively referred patients undergoing a nasal brushing at a national diagnostic centre due to symptoms suggestive of PCD.

Exclusion criteria: Patients who did not wish for their data or surplus samples to be used for research or could not consent because the biopsy was taken elsewhere and couriered to the Royal Brompton Hospital (n=22). Patients who were referred to the service but based on nasal nitric oxide and atypical clinical features further PCD testing was not required (not biopsied n=185).

Diagnosis of PCD

All patients underwent the clinical PCD diagnostic protocol according to standard operating procedures at the Royal Brompton Hospital as follows:

1. Assessment of clinical history for factors suggestive of PCD (n= 378/386; 8 external samples had a limited clinical history).

   Clinical suspicion was considered strong if more than two of the following factors were present and were unexplained by a differential diagnosis such as cystic fibrosis or immune deficiency: Neonatal distress in a term infant, chronic wet cough and/or chronic nasal symptoms since birth, bronchiectasis, situs abnormalities, persistent otitis media, otorrhoea or hearing impairment. Otherwise clinical suspicion was considered modest.

2. Nasal Nitric Oxide measurement – In children over the age of 4yrs able to hold their breath for more than 15 seconds. Four readings from both nostrils taken during a breath hold manoeuvre using a chemiluminescent analyser LR2000 (Logan Research, Rochester, Kent, UK) [9] (n= 129/386). Values of <77nl/min were considered low.

3. High speed video microscopy for cilia beat frequency and ciliary beat pattern measured at 37ºC using a 100x oil objective [10] (n=386/386). If ciliary beat frequency was <8Hz, >16 Hz or the sample contained any area of dyskinesia the sample proceeded to electron microscopy. If ten ciliated strips of more than 10 cells could not be observed the sample was considered inadequate and sample culture was requested. Where culture failed a repeat sample was requested. If 10 strips on 10 cells had completely normal beat pattern and beat frequency the sample was processed for electron microscopy but not analysed (blocked and stored as normal).

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4. Quantitative transmission electron microscopy of ciliary ultrastructure [11] (n=208/386). Electron microscopy was considered normal if >90% of at least 100 cilia from different sample areas showed normal ultrastructure. If less than 100 cross sections could be counted, the sample was considered inadequate and a repeat sample or sample culture was requested. Hallmark defects of PCD were considered to be outer dynein arm defect, inner and outer dynein arm defect, central complex defect, Microtubular disorganisation +/- inner dynein arm defect

5. Air liquid interface culture of unhealthy or inadequate samples with repeat light and electron microscopy [12] (n=115/386)

6. Genotyping from blood samples in patients with likely PCD based on other investigations as part of our genetic research program (n=16/386)

This protocol is standardised and regularly audited across the three UK diagnostic PCD centres and is in accordance with European diagnostic guidelines [5].

Repeat samples were taken following treatment of any upper respiratory tract symptoms when the initial sample was deemed inadequate. Repeat samples were analysed by light microscopy and electron microscopy in the same way as a new case.

A diagnosis of PCD was made following review of all clinical and laboratory findings in a monthly multidisciplinary meeting (MDT) led by a consultant in paediatric respiratory medicine with expert knowledge of PCD. Decision making was based around evidence in recent European Diagnostic Guidelines for the diagnosis of PCD [13].

PCD was diagnosed if the following criteria were met:

1. Clinical symptoms consistent with PCD and if measured low nasal nitric oxide levels (<77nl/min).
2. Abnormal ciliary beat pattern known to be associated with PCD on high speed video microscopy
3. Abnormal cilia ultrastructure demonstrated by transmission electron microscopy and/or unequivocal disease-causing bi-allelic gene mutations recognised to cause PCD.

Patients were considered to be very unlikely to have PCD where there was a:

1. Low or modest clinical suspicion
2. Completely normal ciliary beat pattern OR normal ciliary beat pattern present in the majority of the sample and normal electron microscopy.

Of note, 15 patients in this group had a nasal nitric oxide <77nl/min. In all cases the clinical phenotype was not typical of PCD and this was attributed to another cause of nasal obstruction or poor technique.

In thirty five patients a diagnosis of 'PCD positive' or 'PCD highly unlikely' could not be reached during the study period. Twenty had an insufficient sample from their first or second nasal brushing and have not yet returned for follow up. Fifteen remained inconclusive despite repeating the investigations. These subjects had moderate or high clinical suspicion and an abnormal result on one or more test but did not have a hallmark defect by electron microscopy or an unequivocal gene mutation (where tested). These subjects were excluded from the analysis. None had a beat

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pattern in keeping with a known defect (e.g none were completely immotile or with the hyperfrequent beat pattern associated with DNAH11)

Experimental methods

Nasal brushings were obtained using a modified 3mm cytology brush [13] and the sample applied to the centre of clean uncoated circle slide (Thermo scientific, UK). Six slides were prepared per patient and allowed to dry overnight. All slides were assigned a unique 7-digit code to preserve anonymity and blind the observer to other clinical details of the case. Slides were stored unfixed at -20ºC until immunolabelled. An immunolabelling run was conducted weekly and slides visualised within 48 hours.

After slide preparation for immunofluorescence the remainder of the sample was used for transmission electron microscopy. The impact of splitting the sample on the electron microscopy result was audited regularly and no detrimental effect was found. Conversely there was a slight decrease in the number of insufficient samples compared to the two years prior to the study (21% during study, 37% prior to study). 2013-2015, 37% 2009-2011). 2012 is excluded from this data as samples were used for IF during protocol testing in the run up to the study.

A method adapted from Fliegauf et al was used for immunofluorescence [4]. Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at pH 7.4 for 10 minutes. A combined blocking and permeablisation step using 0.1% Triton in PBS (Triton X-100, Sigma Life Sciences, UK) with 5% milk powder solution was conducted for an hour. This was followed by the addition of primary antibodies for 2

hours and then secondary antibodies for a further 30 minutes. All slides were double labelled with acetylated alpha tubulin (T7451, Sigma Aldrich, dilution1:1000) in order to visualise the cilia. Antibodies of interest were DNAH5 (HPA037470, dilution 1:1000, Sigma Aldrich), DNALI1 (HPA053129, dilution 1:100, Sigma Aldrich), GAS8 (HPA041311, dilution 1:50 Sigma Aldrich), RSPH4A (HPA031196, dilution 1:500, Sigma Aldrich), RSPH9 (HPA031703, Sigma Aldrich, dilution 1:100) or RSPH1(HPA017382, Sigma Aldrich , 1:100). Secondary antibodies used were goat anti-mouse IgG and a goat anti-rabbit IgG (Alexa Fluor 488, A11001 and Alexa Fluor 594, A11012, respectively. Life Technologies, UK). The two secondary antibodies were added simultaneously at 1:1000 dilution in PBS. Thorough washing with PBS was conducted between each step.

Slides were cover-slipped in mountant containing DAPI to preserve the preparation and allow visualisation of the cell nucleus (ProLong Gold P36931, Invitrogen Life Technologies, UK). Preparations were viewed and photographed using a Zeiss Axioskop fluorescent microscope with Axiocam HR camera. Slides were scanned for ciliated cells using a x40 oil immersion objective to identify 488nm labelled acetylated tubulin. In each ciliated cell found, the co-localised 594nm labelled ciliary protein of interest was assessed in a second channel. If there was visual co-localisation of the antibody label with acetylated tubulin the target protein was considered present. If more than seven of ten ciliated cells were clearly labelled the sample was considered normal for that protein. PCD was defined as at least ten of ten ciliated cells with absent or partial staining of the target protein and was repeated on a duplicate slide for confirmation.

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When findings were variable within the sample or the protein of interest was present but difficult to distinguish above the background fluorescence the sample was deemed inconclusive. The sample was deemed insufficient in the absence of 10 ciliated cells.

The discovery cohort showed that diagnostic confidence remained 100% if 100, 75, 50, 25, 10 cells were analysed. It was not deemed appropriate to use less than 10 cells for diagnostic purposes.

Insufficient and inconclusive slides were repeated. If the second assessment was normal the second assessment was included in results (n=71). This is standard practice and repeat assessments are made for all other PCD diagnostic tests if the first result is inconclusive.

Antibodies were used in a two step protocol. All nasal brushings were assessed for DNAH5 (outer dynein arm heavy chain), DNALI1 (inner dynein arm light intermediate chain) and RSPH4A (radial spoke head). A second round of immunofluorescent staining was used in selected cases. GAS8 (nexin - dynein regulatory complex component) was used where light microscopy described the majority of cilia having a stiff or staggered beat or where DNALI1 was absent (n=54). RSPH9 or RSPH1 (alternative radial spoke head proteins) were used when light microscopy described a circular or semi circular beat pattern or RSPH4A was absent or inconclusive (n=17)

Data analysis

The workflow is shown in figure 2. Patients with a diagnosis of 'PCD- positive' or 'PCD-highly unlikely' were included (n=277). Those patients where the diagnosis was inconclusive at the end of the study period were not included (n=35 patients). Those patients where immunofluorescence results were deemed 'insufficient' or 'inconclusive' were also excluded from these calculations (71 patients)

Immunofluorescence result delivery was assessed by time taken to return results, cost of test and procedure failure rate. These factors were compared to electron microscopy as the main confirmatory investigation of structural defect in the cilium.

Cost analysis

The cost analysis for immunofluorescence includes staff salary (0.3WTE per week- 1 day for the processing and half a day for analysis), equipment running costs (fluorescent microscope annual service and bulb replacement) and consumables (primary antibodies, secondary antibodies, slides, coverslips and other reagents). This is based on a throughput of ~200 samples per year.

The costs analysis for electron microscopy includes (1.0 WTE per week – 3 days for processing and sectioning and two days for analysis). Equipment running costs (transmission electron microscope and microtome service) and consumables. This is based on a throughput of ~100 samples per year.

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Neither analysis include initial set up costs such as purchase of equipment. The brushes used for obtaining the cells are not included in either calculation as surplus sample from high speed video microscopy is used.